Concise Review

Latest Developments in Gene Transfer Technology: Achievements, Perspectives, and Controversies over Therapeutic Applications

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ABSTRACT

Over the last decade, more than 300 phase I and phase II gene-based clinical trials have been conducted worldwide for the treatment of cancer and monogenic disorders. Lately, these trials have been extented to the treatment of AIDS and, to a lesser extent, cardiovascular diseases. There are 27 currently active gene therapy protocols for the treatment of HIV-1 infection in the USA. Preclinical studies are currently in progress to evaluate the possibility of increasing the number of gene therapy clinical trials for cardiopathies, and of beginning new gene therapy programs for neurologic illnesses, autoimmuno diseases, allergies, regeneration of tissues, and to implement procedures of allogeneic tissues or cell transplantation. In addition, gene transfer technology has allowed for the development of innovative vaccine design, known as genetic immunization. This technique has already been applied in the AIDS vaccine programs in the USA. These programs aim to confer protective immunity against HIV-1 transmission to individuals who are at risk of infection. Research programs have also been considered to develop therapeutic vaccines for patients with AIDS and generate either preventive or therapeutic vaccines against malaria, tuberculosis, hepatitis A, B and C viruses, influenza virus, La Crosse virus, and Ebola virus. The potential therapeutic applications of gene transfer technology are enormous. However, the effectiveness of gene therapy programs is still questioned. Furthermore, there is growing concern over the matter of safety of gene delivery and controversy has arisen over the proposal to begin in utero gene therapy clinical trials for the treatment of inherited genetic disorders. From this standpoint, despite the latest significant achievements reported in vector design, it is not possible to predict to what extent gene therapeutic interventions will be effective in patients, and in what time frame. *Stem Cells 2000;18:19-39*

INTRODUCTION

The advent of gene transfer technology in therapy marks its tenth anniversary. The first phase I gene-based clinical trial dealt with the treatment of adenosine deaminase deficiency, and is now a milestone of experimental medicine [1]. The initial success of this clinical trial prompted the submission of many other human gene therapy protocols. Over the last decade, more than 300 phase I and phase II gene therapy clinical trials have been conducted worldwide for the treatment of cancer [2-4], and of inherited or acquired genetic disorders [3, 4]. The aim of these clinical trials was mainly to assess the degree of toxicity of the various gene delivery systems and the constructs employed in the study. The possible therapeutic efficacy of the clinical trials was only a secondary issue, which in many cases could not even be determined because of the preliminary nature of the study design. In many cases the cohorts of patients were already terminally ill at the time of the gene therapy intervention, especially where cancer patients were being treated.

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Already in the early 1990s, the first phase of enthusiastic pursuit of gene therapy programs was soon followed by a certain degree of skepticism. The level of vector design development was not adequate enough to meet all the enthusiastic expectations of the investigators who were involved in gene therapy programs at that time [5]. In the following years, research strategies were cautiously planned and an enormous effort was dedicated towards the improvement of vector design [5]. In this matter, significant advances have recently been realized, and they are discussed in this review. The standpoint of the current gene therapy research programs clearly indicates both the presence of a sober optimism among scientists, and a more active role of gene transfer technology in clinical trials [6-9] for the treatment of cancer [2, 3], inherited or acquired monogenic disorders [3], and AIDS [3]. Indeed, gene therapy is one of the fastest growing areas in experimental medicine. As of June 1998, in the USA there were 244 gene therapy clinical trials that were either active or in the evaluation phase by the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) [3]. Most of these trials were either phase I or phase II. In March 1999, the RAC reported that 248 human gene therapy protocols were registrated at the Office of Recombinant DNA Activities. Interestingly, 173 of these trials are for treatment of cancer, 36 for monogenic disorders (mainly cystic fybrosis), 27 for AIDS and 12 for unspecified other disorders (this information is available at http://www.nih.gov/od/orda/). This picture reflects the worldwide trend. This contrasts with the early predictions on the fate of gene therapy, which was thought to be primarily employed to treat inherited or acquired genetic disorders. Such a dramatic change of pattern is certainly motivated by the technical difficulties experienced in establishing long-term transgene expression in humans [10], which is one of the most critical requirements for the successful correction of monogenic disorders [5]. In addition, gene therapy clinical research for the treatment of inherited or acquired genetic diseases is much less likely to receive support from pharmaceutical industries, due to the rarity of these illnesses. Undoubtedly, pharmaceutical companies are interested in developing gene therapy programs for the treatment of more common pathological conditions. This explains, in part, the increase in the number of cancer gene therapy protocols, the beginning of AIDS gene therapy programs, and the sudden development of preclinical studies for gene-based treatments of cardiovascular diseases and neurologic disorders. It has been estimated that about 3,500 patients have been enrolled worldwide in gene-based clinical trials. It is expected that this figure will increase in the next few years. However, the real effectiveness of gene therapy programs is still in question. After a decade of clinical trials, the therapeutic applications of gene transfer technology are still at a rather preliminary stage [5]. Despite the latest improvements reported in the area of vector design, viral and nonviral-based vectors are not sufficiently developed to allow for a large scale application of gene therapy in phase III clinical trials. The purpose of these trials is to assess the therapeutic efficacy of the various treatments in patients. Preclinical studies are currently in progress to test the level of improvement in the various gene delivery systems. The potential applications of gene transfer technology in therapy are enormous. As anticipated, the spectrum range of the pathological conditions that can potentially be treated comprise cancer [2], inherited or acquired monogenic disorders [3, 4], AIDS [3], and other infectious diseases. Most likely, this spectrum will soon be extended to cardiopathies [5, 10, 11], neurologic illnesses [12-14], regeneration of tissues [15-17], and allergies [18]. Indeed, some gene therapy clinical trials have already been carried out for the treatment of cardiovascular diseases [19-21]. In addition, gene transfer technology is emerging as a powerful tool for innovative vaccine design, which has been termed genetic immunization [5, 22-32]. The vectors are either based on viral or nonviral gene delivery systems, and on mechanic administration of naked DNA. The AIDS vaccine programs, which began in the USA in 1996, have widely adopted these gene-based immunization techniques in phase I and phase II clinical trials [22-24, 33], in attempts to confer protective immunity to individuals at risk of HIV-1 infection. Studies are currently in progress to generate more viral vectors to be used in the AIDS vaccine programs [34], and develop therapeutic vaccines for patients with AIDS [35]. Several research programs are addressing the possibility of developing either preventive or therapeutic DNA-based vaccines against malaria [29, 30, 32, 36, 37], tuberculosis [26, 32], hepatitis A, B and C viruses [38-45], influenza virus [27, 28], Ebola virus [31], and La Crosse virus [46]. Interestingly, the same principle of genetic immunization may be used to treat allergies [18] and autoimmune diseases [47, 48], or to prevent the rejection of transplanted allograft tissues [49, 50].

Another line of intervention that has recently been proposed is in utero gene therapy. These clinical trials envisage the treatment and/or prevention of certain inherited genetic disorders, which may have catastrophic outcomes in children. This proposal has raised concern and conflict within the scientific comunity [51-53]. For the moment, the RAC has not authorized in utero gene therapy clinical trials, as there are not sufficient data from animal model systems to ensure the safety of this procedure in humans.

There are several motivations for interest in establishing gene therapy and genetic immunization programs. The pursuit of these programs implies an enormous effort by

scientists and clinicians. Despite the latest progress reported in the area of vector design, research strategies still have to tackle critically important issues, such as further improvement of gene transfer technology, especially for in vivo gene delivery applications, regulation and control of the transgene expression post-cell transduction, and a variety of complex safety matters. These three main issues are to some extent intertwined and pose severe limitations on the applications of gene transfer technology in therapy.

Achievements in Vector Design

The successful realization of gene therapy programs in medicine is highly dependent upon the degree of vector design development. This area of investigation has to deal with a number of pressing and complex issues in order to optimize the performance of gene transfer technology in preclinical studies and clinical research. The aspects that need to be addressed may be summarized as follows:

- The transduction efficiency of both viral- and nonviral-based vectors must be improved. Also, the production and purification procedures for vectors must be optimized.
- In the matter of gene delivery safety, the first rule is that vectors must not be pathogenic or toxic to the patients. For this reason, viral vectors have been engineered to be noncompetent for replication, and devoid of viral factors that may pose a hazard in humans. However, a great deal of attention is still drawn to the possibility of replication-competent virus formation in patients. Another concern is the issue of insertional mutagenesis of vectors based on retroviruses or on adeno-associated virus (AAV) type 1 or type 2. A rather new aspect that has been considered is the possible recombination between retroviral-based vectors and human endogenous retroviruses (HERVs). In order to improve the performance of gene transfer technology, viral-based vectors must be modified in order to reduce their toxicity and immunogenicity in patients. A number of significant advances have been accomplished in this respect. One study has also raised some concern about the immunogenicity of selectable markers [54], which normally derive from bacteria. Therefore, the transduction of cells of the hematopoietic lineages may lead to selectable markers entering the antigen-presenting cell pathway. This in turn would render the transduced cells susceptible to cytotoxic T lymphocyte (CTL) immune responses [54]. Indeed, this principle is the very basis of genetic immunization.
- It is necessary to enhance the targeting and specificity of vectors to avoid unpredictable side effects due to the ectopic expression of the transgene in normal tissues. This requirement is essential to generate gene delivery systems suitable for in vivo administration. Most of the human gene therapy protocols currently rely on ex vivo gene transfer manipulations, in which certain cells or tissues must be removed from the patient, transduced in vitro, possibly selected for the expression of the transgene, and then reinfused into the patient. The entire procedure is costly and distressful for the patient. Health care systems and pharmaceutical companies would greatly benefit from the possibility of applying gene therapy approaches based on in vivo gene delivery, as the therapeutic interventions are minimally invasive, and may only require either an injection or the administration of pills [18]. Indeed, the in vivo transduction approach would also allow for a broader application of gene transfer technology in therapy. Certain pathological conditions cannot be dealt with using the ex vivo gene therapy approach, as not all cells or tissues can be surgically removed. Neurons or cardiac cells are an example. However, the in vivo gene therapy approach poses many additional safety concerns versus the ex vivo one. Recent studies have shown there is a possibility that exogenous DNA (transgene and/or viral vector sequences) may eventually be transmitted to the germ line through systematic in vivo administration of viral vectors [55, 56]. Sensitive nested polymerase chain reaction (PCR) techniques have allowed for the detection of low levels of exogenous viral vector DNA in the ovaries and testes of mice, which received systematic administration of adenoviral vectors [56]. Ninety-four percent of these animals tested positive for the presence of adenoviral DNA in the gonads. However, after mating the animals there was no evidence of germ line transmission of adenoviral DNA in the offspring [56]. This issue should also be addressed for in vivo retroviral- or AAV-mediated gene transfer. These viral vectors may have higher probabilities of entering the germ line, as they integrate their chimeric viral genome into host chromosomal DNA [5].
- In many cases, the possibility of regulating transgene expression following cell transduction would be a highly desirable feature. This should allow for the activation of a transgene when it is needed, the maintenance of transgene expression within a therapeutic window, and the possibility of silencing a transgene if necessary. There have been a number of attempts to generate inducible systems. Partial successes have been reported in the in vitro system [5, 57-62] and animal models [63-67]. However, whether transgene

regulation can be achieved in patients is still an open question.

- The possibility of combining gene-based interventions with other therapeutics has to be considered.

A broad arsenal of gene transfer systems is currently avalaible [5] and is still in expansion. The characteristics of the main vector systems are described in Table 1. Each gene delivery system has distinct characteristics and preferential applications in therapy [5]. The vectors that have already been applied in clinical trials are based on retroviruses [68-72], adenovirus [73-78], AAV [79-85], vaccinia virus [86, 87], canarypox virus [87], herpes simplex virus (HSV) [88], cationic liposomes [89-92], polylysine-DNA complexes [93, 94], and injection of naked DNA [22, 26, 27, 30]. As anticipated, the pathological conditions with which gene therapy has dealt so far comprise: cancer [2], inherited or acquired monogenic disorders [3, 4], AIDS [3], and cardiovascular diseases [19-21]. In addition, vectors based on vaccinia virus, canarypox virus, injection of naked DNA and other nonviral vectors have been used in the AIDS vaccination programs in the USA [22-24]. Interestingly, viral-based vectors have also been directly administered to patients in order to transduce in vivo cells that are capable of processing the transgene through the antigen-presenting cell pathway. In these cases, the transgene encodes for certain HIV-1 components. The intracellular expression of viral antigens within transduced cells facilitates the cells' antigen-presenting mechanism. In this way various viral epitopes are associated with host HLA class I antigens and expressed on the cell membrane to elicit the host's CTL immune responses [5].

Preclinical tests have been carried out to characterize the gene delivery properties of vectors based on foami virus [95-97], lentiviruses (such as HIV-1 [98-104] and feline immunodeficiency virus (FIV) [105-107]), human cytomegalovirus (CMV) [108], Epstein-Barr virus [109], negative-strand RNA viruses (influenza virus) [110], alphaviruses [111], herpesvirus saimiri [112], hybrid adenoviral/retroviral vector systems [113, 114], and hybrid alphavirus/retroviral vector [115]. Other preclinical studies are also assessing the level of vector design improvement that has been reported for a variety of gene transfer models.

Vector Systems Based on Retroviruses, Lentiviruses and Foami virus

Retroviruses have attracted a great deal of interest from the standpoint of gene transfer applications [5]. Such interest is certainly motivated by the characteristics of the biology of retroviruses, which belong to the genera of the *retroviridae*. This category also comprises lentiviruses and foami viruses. The *retroviridae* have a long history of cross-species infections [116, 117]. They have been responsible for many zoonotic events (transmission of infectious agents from animals to humans) [116] which indicates that they may be suitable for DNA delivery into humans. The retroviral genome is relatively simple [118], so it may easily be rearranged to generate recombinant viral vector particles which are noncompetent for replication [5], and which can sustain only one round of infection. Retroviral vectors are mainly based on the amphotropic Moloney murine leukemia virus (MLV) [118], and have been used in many gene therapy clinical trials for the treatment of cancer [2, 5], inherited or acquired monogenic disorders [5], and AIDS [119-124]. Lentiviral vectors are based on HIV-1 [98-104] or on FIV [105-107]. Neither lentiviral- or foami virus-based vectors have been used in clinical trials yet. However, the HIV-1-based lentiviral vector system is unlikely to be approved for clinical trials for a variety of reasons. First is the issue of the serum conversion of the patients to HIV-1. Secondly, is the production and administration of lentiviral vector stocks require category three facilities. Third, the large quantities of lentiviral vector stocks that have to be produced for the clinical trials pose an additional concern in the matter of biosafety. Fourth, this vector system is already obsolete, due to the development of the FIV-based lentiviral vector system, which has circumvented all the above-mentioned issues. In fact, FIV has been certified for category two manipulations, and is based on a lentivirus which cannot infect humans. Therefore, the serum conversion to FIV does not raise any concern. The characteristics of the *retroviridae* vector systems are summarized in Table 1. All these viral vector systems can be produced at relatively high titers (10^6-10^7 cfu/ml) [5]. A property of retroviruses is that they can only infect dividing cells, as they need the breakdown of the nuclear membrane to be able to deliver the preintegration complex into the cell nucleus [125]. Conversely, lentiviruses [98-107] and, to a lesser extent, foami viruses [95-97, 126] can also infect nondividing cells. The requirement for active cell division can be either an advantage or a drawback for retroviral vectors. The selective transduction of dividing cells makes retroviral vectors suitable for cancer therapy [5]. On the other hand, retroviral vectors cannot be used for a variety of therapeutic applications, such as neurologic diseases and a number of genetic diseases that require the transduction of hepatocytes [127], as neurons and hepatocytes do not divide. In all these respects, FIV-based lentiviral vectors may find useful applications. Indeed, retroviral vectors have been used in a number of preclinical studies for liver-directed gene transfer and in some clinical trials [127]. The procedure used was based on ex vivo or in vivo transduction of hepatocytes, which were induced to proliferate by complex and artificial procedures [127]. Retroviral-mediated ex vivo transduction relies on stimulating cell division by culturing primary hepatocytes in appropriate

Table 1. Description of the main gene delivery systems

Vectors	Characteristics	Disadvantages and possible adverse effects in therapy
Retroviruses	• Relatively high titers (10^6-10^7 cfu/ml) • Broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • No toxic effect on infected cells • Total insert capacity in the virion is in the range of 10kb (transgene + transfer vector) • They only infect dividing cells	• Random insertion of viral genome, which may possibly result in mutagenesis • Possibility of replication competent virus formation by homologous recombination • Retroviral vector particles are rapidly degraded by the complement • Possible recombination with human endogenous retroviruses (HERVs)
HIV-1-based lentivirus	• It can infect nondividing cells • It can be pseudotyped with retroviral or VSV G envelopes, therefore it also has broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Relatively high titers (10^6-10^7 cfu/ml) • Total insert capacity in the virion is in the range of 10kb (transgene + transfer vector)	• Serum conversion to HIV-1 • Possible proviral insertional mutagenesis in target cells • Presence of tat and rev regulatory proteins, (the early lentiviral vectors also have some HIV-1 accessory proteins) • Possible recombination with human endogenous retroviruses (HERVs) • Possibility of replication competent virus formation by homologous recombination
FIV-based lentivirus	• It can infect nondividing cells • It can be pseudotyped with retroviral or VSV G envelopes, therefore it also has broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Relatively high titers (10^6-10^7 cfu/ml) • Total insert capacity in the virion is in the range of 10kb (transgene + transfer vector)	• Possible proviral insertional mutagenesis in target cells • Presence of FIV regulatory proteins in the early vectors • Possible recombination with human endogenous retroviruses (HERVs) • Possibility of replication competent virus formation by homologous recombination
Foami virus (human or simian)	• It infects dividing cells; nondividing cells are also infected, but to a lesser extent • Foami virus is resistant to lysis mediated by complement • It can be pseudotyped with retroviral or VSV G envelopes, therefore it also has broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Relatively high titers (10^6-10^7 cfu/ml) • Total insert capacity in the virion is in the range of 14kb (transgene + transfer vector)	• Possible proviral insertional mutagenesis in target cells • Possible recombination with human endogenous retroviruses (HERVs) • Serum conversion to foami virus. A number of zoonotic events have occurred among animal care workers, and, so far, there is no evidence that the foami virus is pathogenic in humans, and that it can be transmitted among humans • Possibility of replication competent virus formation by homologous recombination
Adenoviruses	• Very high titers (10^{12} pfu/ml) • Transiently high levels of gene expression • They can also infect nondividing cells • Large DNA inserts can be accommodated in the vector (7-8kb can be simply added to the adenoviral vector; larger DNA inserts can be added, provided that an equivalent part of the viral genome is properly deleted)	• Host immune responses: inflammatory and toxic reactions in patients and depletion of transduced cells • Host's humoral immune responses may neutralize adenoviral vector particles during, or even before, the gene transfer processes • Not suitable for long-term expression of the transgene due to the lack of integration into host genome • Complicated vector genome
Adeno-associated viruses (AAV)	• Wide range of cells can be infected, including cells which do not divide • High titers (10^{10} cfu/ml) • Ability of the virus to establish latent infection by viral genome integration into cell genome • Viral integration specific for human chromosome 19 (only for wild-type AAV) • Nonpathogenic, nontoxic • Small genome (5kb)	• High titers of pure virus are difficult to obtain • AAV requires a helper adeno- or herpesvirus • This vector system is still not well characterized • Limited capacity for foreign genes (about 4kB) • Lack of specific integration for recombinant AAV vectors, which may possibly result in cell mutagenesis
Herpes simplex virus (HSV)	• Titers are in the range of 10^4 to 10^8 cfu/ml • The maximum size of the transgene can reach 30kb • It does not integrate into the cell genome • It allows for long-term expression of the transgene in neuronal cells • It induces cytopathic effects in cancer cells	• Host immune responses, inflammatory and toxic reactions in patients • Complicated vector genome
Cationic liposomes or DNA-protein complexes	• They are not infectious • Theoretically, there is no limit to the size of DNA • They are suitable for oligonucleotide delivery • Low degree of toxicity	• Targeting is not specific • Low transfection efficiency • Only transient expression • Difficult in vivo applications • Host immune responses, inflammatory reactions in patients if they express chimerical cell receptors on their surface, or in the presence of unmethylated CpG sequences of bacterial plasmid DNA

media [127]. This approach has been employed in preclinical studies for the following genetic diseases: type I tyrosinemia, familial hypercholesterolemia and α_1-antitripsin deficiency [127]. One clinical trial was conducted to treat familial hypercholesterolemia by retroviral-mediated ex vivo gene transfer. The low-density lipoprotein (LDL) receptor gene was introduced into hepatocytes that had been surgically removed from patients, and which were then reinfused into the liver following gene transduction [128, 129]. The procedure was safe but there was no convincing evidence of therapeutic efficacy [127]. Liver biopsies were removed after treatment, and few cells tested positive for the expression of LDL-receptor [127], indicating that the transduction efficiency was not high, or that transduced cells were lost or eliminated after reinfusion into the liver. In vivo retroviral-mediated transduction of hepatocytes is even more complicated, as it requires artificial regeneration of the liver [127]. This may be achieved by a variety of means: partial hepatectomy, chemical injury, administration of growth-stimulating drugs or vascular occlusion [127]. Experiments in animal models have shown efficient retroviral-mediated gene transfer into the liver of rodents [127], but a poor efficacy of intervention in larger animals such as dogs [127]. This is probably due to the different kinetics of liver regeneration between large mammals and rodents. In conclusion, in vivo administration of retroviral vectors into the liver does not seem applicable to humans. Probably, the development of a retroviral vector system based on the hepatitis B virus may facilitate liver-directed gene delivery. In this respect, a hepatitis B-based retroviral vector is under development [130, 131]. Interestingly, one study has shown successful liver-directed hepatitis B viral-mediated gene transfer of green fluorescence protein. In addition, the delivery of type I interferon by hepatitis B-based retroviral vector has suppressed endogenous wild-type virus replication in the duck model of hepatitis B virus infection [131]. However, this viral vector system needs further characterization, and should also be adapted to the rodent animal model before considering its application in clinical trials.

All the viral vectors based on *retroviridae* can be used to transduce a wide range of cell types. This is due to the fact that HIV-1, FIV and foami virus cores can be pseudotyped with the MLV amphotropic envelope or vesicular stomatitis virus G (VSV G) glycoprotein (Table 1) [132, 133]. Pseudotyping with the VSV G glycoprotein also allows for easy purification of the various viral vector particles, as they became more stable and resistant, so they can be isolated from the cell culture supernatants by simple ultracentrifugation [134]. Foami viral vectors have a broad cell tropism, even without being pseudotyped with MLV amphotropic envelopes or with VSV G glycoprotein [95-97, 126]. Interestingly, wild-type foami viruses are resistant to complement-mediated lysis [95] and have a total insert capacity in the virion of approximately 14kb [95]. Conversely, MLV-based retroviral, lentiviral and foami viral vectors pseudotyped either with amphotropic retroviral envelopes or VSV G glycoprotein are susceptible to complement-mediated lysis [135-138] and their total insert capacity in the virion is in the range of 10kb [5]. It has been demonstrated that packaging cell lines expressing galactosyl(alpha1-3)galactosyl (alphaGal) sugars generate enveloped viruses that are more susceptible to complement attachment [136]. The viral systems analyzed in this study were based on VSV, HIV-2 and human foami virus [136]. It has been argued that the humoral immune response to alphaGal may be a mechanism of defense against the transmission of viral agents from animals to humans [136], and that viral vectors for human gene therapy should be produced from alphaGal-negative cells [136]. Another study has reported the production of MLV-based amphotropic retroviral vectors resistant to human complement [139]. This was achieved by expressing hybrid amphotropic envelopes on the viral membrane. These hybrid amphotropic envelopes were generated by fusing in frame the catalytic domain of the human complement regulatory protein decay-accelerating factor with a portion of the envelope [139].

The possibility of concentrating retroviral, lentiviral, and foami viral vector particles may improve the transduction efficiency for both ex vivo and in vivo applications. The protection from complement-mediated lysis is particularly required for the optimization of in vivo gene transfer models. A number of other studies have been conducted to further improve the performance of retroviral vectors in preclinical studies and clinical trials. A simple approach consists of using enhanced green fluorescence protein as reporter gene [140-143]. This allows for the rapid detection and isolation of the fraction of cells that have been transduced ex vivo. In addition, the green fluorescence protein can be readily detected in tissues following infusion of transduced cells into the animals [140, 141]. Other strategies to improve the retroviral transduction efficiency are based on the artificial induction of cell division. This can be achieved in many ways: preincubation of primary cultures of hematopoietic stem cells with various interleukins (IL-2, IL-3, IL-6) and/or other growth factors or colony-stimulating factors [144-146]; combination of retroviral- and lipofectAMINE-mediated gene transfer into stem cells prestimulated with IL-2 (in this study, lipofectAMINE was used to facilitate the delivery of retroviral vectors into the target cells) [147]; colocalization of retroviral particles and hematopoietic stem cells on specific fibronectin fragments (Retronectin) [148]; combination of Retronectin system with prestimulation of hematopoietic stem cells with ILs or other growth factors [149, 150]. Ex vivo retroviral transduction of human hematopoietic stem cells also has several disadvantages. Besides being costly and time-consuming, this

approach may introduce some artifacts into hematopoietic stem cells. For instance, the in vitro culture conditions may impair the ability of transduced hematopoietic stem cells to engraft once they are reinfused into the subject. This situation has already been mentioned for the gene-based clinical trial for the treatment of familial hypercholesterolemia, in which the target cells were hepatocytes [127]. The tissue culture conditions for the ex vivo propagation and transduction of human hematopoietic stem cells are conducted at nonphysiologic cell concentrations, and require the combination of growth factors that may induce cell differentiation and, therefore, pose a limitation to the long-term engraftment of the transduced cells. It has been observed that HIV-1- and FIV-based lentiviral vectors may be more suitable for the transduction of hematopoietic cells than amphotropic retroviral vectors [98, 100, 104, 107]. The ability of lentiviruses to also infect nondividing cells may circumvent the issue of prestimulating hematopoietic stem cells [151]. Moreover, lentiviruses usually yield higher transduction efficiency of primary stem cell cultures than retroviral vectors [152, 153]. However, an important aspect that must be addressed in the matter of lentiviral-mediated gene transfer is to establish whether the transfer vector remains episomal in the nucleus of transduced cells that are in G_0 phase. Transgene expression detected following lentiviral transduction of quiescent cells may indeed derive from extrachromosomal double-stranded DNA transfer vector. If this is the situation, lentiviral transduction of quiescent cells may only allow for transient expression of the transgene.

An important safety issue in the matter of viral-mediated gene transfer is the formation of viral-competent viruses in patients, which may occur by homologous recombination events within the packaging cell lines. Retroviral vector stocks are routinely monitored in clinical trials for the absence of replication-competent retroviruses (RCR) [154]. The techniques are essentially based on sensitive PCR and serological enzyme-linked immunosorbent assay [154]. In addition, retroviral stocks must be tested for the absence of endotoxins and various contaminating agents, such as bacteria and fungi, which may be acquired during the propagation of packaging cell lines or target cells [119, 155]. The purity of the various genetic material used in the trial must also be tested [119, 155]. The RCR formation is a rather unlikely event due to the design of retroviral vector. The current trend is to produce high titer retroviral vector stocks transiently [5] in order to further minimize the possibility of recombination events among the various retroviral components in the packaging cell line. These transient systems are based on three plasmid cotransfections of the highly transfectable 293T cell line [156]. As reviewed elsewhere [5], the proviral genome has been broken down into three parts, and overlapping sequences have been mostly removed. The RCR formation is unlikely due to the fact that it would require simultaneous rearrangement among three different plasmids in a specific configuration in a very limited period of time. The transfection procedure usually takes between 48 to 72 h to produce the retroviral vector stocks [5]. So far, the retroviral vectors used in clinical trials derive from conventional packaging cell lines, which were previously approved for clinical applications by the U.S. Food and Drug Administration [3, 157]. Studies are currently addressing the issue of generating clinical grade retroviral vector stocks by transient transfection systems [158].

Another safety concern is the possible recombination between retroviral vectors and HERVs in patients (Table 1). The human genome contains thousands of HERV sequences [159-161], most of which are defective genes. These HERV sequences derive from ancient retroviral infections [160] in which transmission occurred either in germ line cells or cells in the early embryo [160, 161]. About 1% of the human genome is composed of HERV-related sequences [161], and probably more than 10% of the human genome may have evolved through reverse transcription mechanism [161]. So far, only one HERV has been found that encodes for a complete viral particle, which was named HERV-K [162]. However, HERV-K is not competent for replication [162]. The biological relevance of HERVs deserves further investigation. HERVs have some possible advantageous effects in fundamental biological processes such as: development and/or differentiation, protection from superinfection by exogenous retroviruses, protection of the embryo from retroviral infection (germ line vaccination), cell fusion, tissue-specific gene expression, alternative splicing, and polyadenylation [161]. The potential pathogenicity of HERVs cannot be predicted. They may be involved in the development of malignancies and autoimmune diseases [161]. The envelope of an HERV may either protect the host from exogenous retroviral infection in a receptor interference fashion [163] or dysregulate the local cellular immunity through a superantigen-encoded region, as proposed for type I diabetes [164]. A study has observed that the multiple sclerosis-associated retrovirus detected in the plasma of patients with multiple sclerosis [165, 166] has high homology to an HERV [167], which was named HERV-W. Xenotransplantation techniques and gene therapy approaches based on *retroviridae* vectors may eventually tamper with the biology of HERVs [161]. Retroviral vectors may recombine with HERVs in patients, and generate a variety of possible adverse effects. At this point in time, we cannot predict possible adverse effects of recombination due to the lack of sufficient information about HERVs. What one can expect is the formation of RCR in patients, or the expression of HERV genes that were silent prior to gene therapy or xenotransplantation intervention. If such events should occur, most likely the subject may develop cancer or become susceptible to immune system dysregulation.

The integration of the retroviral genome into chromosomes allows for stable transgene expression. This stability is also due to the low degree of retroviral particle immunogenicity. This is in contrast with what has been observed for adenoviral-mediated gene transfer, where transgene expression is only transient. There are two reasons for the transient nature of transgene expression in adenoviral-mediated gene transfer. First, the adenoviral genome does not integrate into the host chromosomal DNA [5]. Second, the adenoviral particles are immunogenic [5] and express leaky adenoviral genes that render the transduced cells susceptible to CTL immune responses [168-171]. Stable retroviral-mediated transgene expression is desirable for the treatment of diseases that require long-term expression of the transgene, such as genetic disorders and neurologic illnesses [5]. However, the duration of transgene expression is still not optimal. This is because the retroviral long terminal repeats (LTRs) are susceptible to methylation in CpG-rich islands, which may silence the gene transcription [172, 173]. The incidence of this phenomenon depends on the type of transduced cells and the site of retroviral genome integration [174]. It has been shown that Sp1 binding sites may, to some extent, prevent the methylation of the promoter [175]. Retroviral vectors based on murine embryonic stem cell virus (MESV) [176, 177] and on murine stem cell virus (MSCV) [178] have been engineered to optimize the duration of transgene expression in undifferentiated murine embryonic and hematopoietic cells [176-178]. To this end, the LTRs of the MESV- and of the MSCV-based vectors have been modified. In the MESV vectors, the 5′-LTR contains an extra Sp1 binding site, which has been introduced by a point mutation [176, 177]. This has optimized, to some extent, the duration of transgene expression in embryonic and hematopoietic cells. However, silencing of transcription has been observed following the differentiation of embryonic stem cells [179]. The MSCV-based vectors, in addition to the point mutation that creates an Sp1 binding site, contain another point mutation that destroys the binding site of the embryonal LTR-binding protein (ELP) [178]. ELP is a transcriptional suppressor of the activity of the MLV 5′-LTR in undifferentiated murine embryonal carcinoma cells [180]. These modifications have further improved the performance of retroviral vectors in terms of duration of transgene expression. However, better evaluation of the exact extent of this improvement in in vivo systems is needed.

The random insertion of the retroviral transfer vector has several drawbacks: it may damage the cell genome, cause the inactivation of tumor suppressor genes, or induce the expression of cellular oncogenes. Probably, this is not sufficient to generate a neoplasia, as cancer is a multistep process which requires a combination of genetic alterations and the expression of cellular and/or exogenous oncogenic factors [181]. However, if the transduced cells should be genetically impaired by the random insertion of the viral vector's genome, this would at least predispose the cells to undergo to neoplastic transformation. To date, human gene therapy protocols have been applied only to a limited number of patients, and most of them did not have a long life expectancy. An important question is what happens if retroviral-mediated gene transfer is applied to larger scale clinical trials and subjects who have a life expectancy in the range of some decades? The current development of preventive cancer prognosis cannot answer this question, so it is not possible to properly assess the ratio of benefit to risk for all the patients. A recent study has addressed the issue of cell transformation induced by retroviral-mediated gene transfer in an in vitro system [182]. Mouse fibroblasts BALB/c-3T3 cells were transduced with a retroviral vector, and the transformation frequency was compared to that of the untransduced cells [182]. The parental cell line undergoes spontaneous transformation that is in the range of 1.1×10^{-5} [183]. In this study, the transformation rate of retrovirally transduced BALB/c-3T3 cells was in the same range [182]. The number of integrated proviral copies per cell genome varied from one to six, depending on transduction efficiency [182]. So, improved transduction efficiency is correlated with better transgene expression, which in turn is due to the higher number of integrated retroviral transfer vector's copies per cell genome. But this is also proportional to the higher risk of mutagenic events. Previous studies on retroviral-induced mutagenesis in mammalian cells have found a ratio of "mutations versus insertional events" which ranged from 10^{-9} to 10^{-3} [184-189]. Such variability indicates that the ratio of mutations per insertional events depends on the cell type and assay system. This ratio should be established for human primary lymphocytes, which normally are not retrovirally transduced as efficiently as mouse fibroblasts [182, 190, 191] or other cultured cell lines [191]. However, the lower transduction efficiency, per se, does not guarantee a lower ratio of "mutations versus insertional events" in human primary lymphocytes. All these findings suggest that in the design of clinical protocols using retroviral-mediated gene transfer, the average number of integrated viral genomes should be carefully evaluated. Such a procedure is feasible for ex vivo retroviral-mediated gene transfer, but not for the in vivo administration system.

Overall, the in vivo administration of retroviral vectors poses a number of additional safety concerns and technical limitations if compared to the ex vivo gene transfer model. To pursue the goal of safe and efficient in vivo retroviral transduction, it is necessary to generate tissue- or cell-specific retroviral vectors, which can integrate their genome in

safe cell chromosomal sites. The latter issue has never been tackled, whereas the engineering of ecotropic-based retroviral vectors with altered cell tropism has attracted much attention [5], but all the attempts had little success. The chimeric retroviral particles that have been produced have a low transduction capacity [5], or even fail the gene transfer process [192]. To date, the ex vivo retroviral-mediated gene transfer model is more realistic than the in vivo one, although it is not optimal for gene therapy applications. Also, from the standpoint of safety concern, the ex vivo procedure can be more easily monitored.

Adenoviral Vectors

There has been a remarkable increase in gene therapy clinical trials based on adenoviral-mediated gene transfer over the last three years [193]. For instance, of 170 gene therapy clinical trials registered with the NIH RAC in 1996, only 15% relied on adenoviral vectors [193]. In the following two years, 91 new human gene therapy protocols were submitted to the NIH RAC, and 32% of them were based on adenoviral-mediated gene transfer [193]. This finding reflects the improvement in adenoviral vector design, which has allowed for a wider application of adenoviral-mediated gene transfer in preclinical studies and therapy. As previously reviewed [5], the first recombinant adenoviral vectors were engineered in 1985, and were based on serotype 2 or 5 [73-75], due to the fact that these two adenoviral serotypes are not associated with severe illnesses and do not cause tumors in animals, in contrast to the other serotypes. The first applications of adenoviral vectors in gene therapy clinical trials were conducted in the early 1990s for the treatment of patients with cystic fibrosis [194]. Now, adenoviral vectors are widely employed in human cancer gene therapy [2] and some other somatic gene therapy clinical trials [195]. Adenoviral vectors have a number of positive characteristics (Table 1): they can also transduce nondividing cells; they can be produced at very high titers of 10^{10} pfu/ml, and easily concentrated to 10^{12} pfu/ml; it is possible to achieve high levels of transgene expression, but only transiently; and they have a large insertional capacity for foreign genes, which is in the range of 7-8 kb (about 6% of the wild-type adenoviral genome). If the genome of the adenoviral vector is properly deleted, it is possible to accomodate into the virion DNA fragments even bigger than 7-8 kb, depending on the size of the deletion [5]. In addition, adenoviral vectors based on serotype 5 may be excellent for liver-directed gene therapy approaches, as the adenovirus serotype 5 is preferentially localized in the liver post-intravenous injection of rodents [196, 197]. All these properties make adenoviral vectors a very attractive gene delivery system, which could potentially be employed in a variety of pathological conditions, such as neurologic disorders, cardiopathies, inherited or acquired monogenic diseases, and cancer. Unfortunately, the current design of adenoviral-mediated gene transfer is affected by some drawbacks, which severely limit the applications of adenoviral vectors in preclinical studies and therapy (Table 1). Firstly, adenoviral vector particles are highly immunogenic in the host [5]. This is a significant obstacle to improving adenoviral vector design. Besides eliciting inflammatory and toxic reactions in the host, immunogenicity is also responsible for the depletion of adenovirally transduced cells [198-203], and may also reduce the efficacy of adenoviral-mediated gene transfer readministration to the patients. In fact, adenoviral particles are also susceptible to humoral immune responses, which may neutralize most of the vector load before the gene transfer is carried out [204]. The issue of humoral immune responses is even more complicated in patients, as, in most cases, they already have an acquired immunity to adenoviruses prior to the gene therapy intervention. Immunogenicity derives from leaky expression of adenoviral early genes (E1, E2, E3 and E4) following adenoviral cell transduction [5]. The deletion of E1 gene is essential for generating replication-defective adenoviral vectors [205]. E1 functions are supplied in trans by a cell line that is stably transformed with the adenoviral E1 gene [206]. Secondly, the transgene expression can only be transient, because adenoviruses do not integrate their genome into the cellular chromosomial DNA [5, 207]. If such an event should occur, it would just be fortuitous [204]. Therefore, both immunogenicity and lack of adenoviral genome integration into the host's chromosomal DNA contrive to suppress long-term transgene expression of adenoviral-mediated gene transfer. Taken together, these properties limit the application of adenoviral-mediated gene transfer for the treatment of pathological conditions that require a long-term transgene(s) expression, such as inherited or acquired monogenic disorders, neurologic illnesses and cardiovascular diseases. From the standpoint of gene-based cancer therapy, adenoviral vectors are useful, provided that inflammatory and other toxic reactions in patients are carefully monitored. One goal of cancer therapy is the selective destruction of neoplastic tissues and cells. This may be accomplished by a variety of means [2], and on a transient basis, followed by systematic administration of adenoviral vectors carrying the appropriate transgene. Another advantage of adenoviral vectors in cancer gene therapy is that they can transduce neoplastic cells regardless of their mitotic status. In most of the cancer gene therapy clinical trials, adenoviral vectors have been administrated in vivo [2], and have been used to deliver drug-sensitivity genes, such as the herpes virus timidine kinase [2, 208-210], immunomodulators such as IL-2 [2, 211], melanoma tumor antigens, such as MART-1 [2] or gp100 [2], or tumor suppressor genes, such as *p53* [2, 212, 213]. The neoplasias that have been treated so far

with adenoviral-based gene transfer include: melanoma [2], prostate [2, 209], mesothelioma [208, 210, 214], metastatic colorectal carcinoma in the liver [213], lung cancer [212], neuroblastoma [2], glioblastoma [2], ovarian cancer [2], and squamous cell carcinoma of the head and neck [2].

Although adenoviral vector technology has been considerably improved in terms of gene transfer applications, the main issue of vector design remains the avoidance of immune responses. This problem has been tackled from different angles. A number of studies have designed new generations of adenoviral vectors. In this respect, two types of approches have been pursued: deletion of E1, E2 and E4 genes in order to avoid the expression of immunogenic viral proteins within transduced cells [127]; and overexpression via a strong constitutive promoter of the E3-encoded 19 kDa glycoprotein (gp19K) in adenoviral vectors lacking the E1 gene [215, 216]. The latter approach has provided encouraging results in terms of more stable transgene expression in the liver [215, 216] or in the lung of rodents [215]. The function of the E3-encoded gp19K is to inhibit the transport of the major histocompatibility complex class I molecules to the cell membrane [216]. This results in impairment of the antigen-presenting cell mechanism, which avoids the clearance of adenovirally transduced cells by CTL immune responses [217, 218].

Adenoviral vectors lacking E2a function and the E1 gene have allowed for a prolonged but transient, transgene expression in the liver of mice [219], and in correcting the phenotype of ornithine carbamyl-transferase deficiency in a murine model [220]. E2a gene expression was neutralized by mutations that rendered the adenoviral protein temperature-sensitive [219, 221]. The combination of E1 and E4 adenoviral gene deletions has required the construction of helper cell lines to supply the E4 functions [222, 223]. The results that have been obtained in the animal model are rather contradictory. One study has shown significantly longer transgene expression for the double E1 + E4 deletion compared to the single E1-deleted adenoviral vector system [224], if the transgene is not per se immunogenic [225]. Conversely, other studies comparing the double E1 and E4 deletion and the single E1-deleted adenoviral vector systems have either reported no substantial benefit in removing the E4 gene [223], or even a detrimental effect on the duration of the transgene expression for the E1 and E4 deficient system [227]. A variety of factors may be involved in the generation of such contrasting results [127]. One could be the innate immunity of the animal model used in the study [228], or the route of adenoviral vector administration [229], which may also cause the depletion of the adenoviral genome. This underlines the importance of designing and conducting in vivo studies under more uniform conditions in order to avoid dramatic disparities of results among different groups of investigators. However, if minor differences in the modality of administration of adenoviral vectors and genetics of the animal models affect the results of the studies, even greater difficulties should be expected in applying adenoviral vectors in clinical trials. Studies are still in progress to further characterize and evaluate the beneficial role of sequential and combined deletions of early adenoviral genes in terms of suppression of immunogenicity related to adenoviral-mediated gene transfer. Interestingly, recent reports have revealed that open reading frame 3 (ORF3) of the gene E4 is required for the persistent expression in vivo and in vitro of a transgene regulated by an internal CMV promoter [230, 231]. In one study, the adenoviral vector system lacks E1, E2a, E3 and E4 except the ORF3 [231], whereas the other study has addressed the functions of ORF3 and of ORF4, ORF6 and ORF6/7 of E4 gene in the context of an adenoviral vector lacking E1 and E3 [230].

Other strategies have been considered in order to minimize the adverse effects of immune responses related to adenoviral-mediated gene transfer. Basically, these alternative approaches consist of reducing the administration load of the adenoviral vector into the patients, by developing high-efficiency transgene expression vectors combined with short-term immune suppression of the subject [203, 232], and/or by generating chimeric adenoviral capsids, with the intent of enhancing the binding affinity for the target cells [5]. This may be achieved by different means. One possibility is the engineering of an adenoviral vector type 5 carrying the fiber genes of the adenovirus type 7 [233, 234]. This chimeric capsid has an enhanced binding affinity for human lung epithelial cells [233, 234], but it is not suitable for efficient liver-directed gene transfer. An interesting observation is that the fiber swapping between adenovirus type 5 and type 7 may also affect the intracellular trafficking of the adenoviral transfer vector [234]. Another chimera was generated by engineering an adenoviral vector type 2 expressing the fiber gene of the adenovirus type 17, in order to optimize targeting to human airway epithelium [235], which is refractory to the adenovirus serotype 2 infection. It has been demonstrated that efficient adenoviral-mediated gene transfer to the human airway is contingent upon $\alpha V\beta 5$ integrin expression [236]. Another possibility that has been explored is the production of adenoviral vector particles with altered cell tropism, in which exogenous genes are introduced into the adenoviral capsid [237-242]. In one of these studies, the insertion of an Arg-Gly-Asp (RGD) motif into the fiber gene of an adenoviral vector has generated a chimeric vector of expanded cell tropism, and with enhanced transduction efficiency for primary tumor cells [238]. Another study has shown a remarkable increase of adenoviral transduction of muscle cells by fusing in frame a polylysine moiety to the fiber protein of the adenoviral capsid [243]. Other attempts to optimize the adenoviral transduction in vivo consist of combining

adenoviral vectors with nonviral gene delivery systems, such as: lipofectAMINE to enhance the transduction of human T lymphocytes [244] (an analogous approach has already been mentioned for retroviral vector transduction [147]); poloxamer 407 to facilitate percutaneous adenoviral-mediated gene transfer [245]; and polyethylene glycol (PEG)ylation of adenoviral particles to protect them from neutralizing antibodies both in vitro and in vivo systems [246]. The latter study has brilliantly addressed the issue of humoral immune responses to the readministration of adenoviral vectors in the murine model [246]. In this study, the adenoviral capsid has been masked by PEG to prevent the attachment of neutralizing antibodies. PEG has been covalently bound to the capsid via activated tresyl-monomethoxypolyethylene glycol, which preferentially reacts with the amino terminal of lysine residues [246]. These PEG-modified adenoviral vector particles were efficiently protected from humoral immune responses after readministration to the lungs of mice [246]. A recent study has addressed the issue of improving the long-term expression of adenoviral vectors by engineering a hybrid adenoviral/AAV vector system [247]. This approach parallels the previously mentioned adenoviral/retroviral hybrid vector system [113, 114]. The difference is that the adenoviral vector delivers into the animal an AAV-based vector that also can transduce nondividing cells [247].

Other safety issues related to adenoviral-mediated gene transfer are related to the possible formation of a replication-competent adenovirus, and to the previously mentioned dissemination of adenoviral vectors to the gonads, which may potentially result in trasmission to the germ line [56]. A study has raised an interesting point about the possible higher probability of replication-competent adenovirus formation in large scale production of adenoviral vectors [248].

AAV-Based Vector

AAV is a nonenveloped human single-stranded DNA virus that belongs to the genera of the *Parvoviridae* and does not seem to be associated with any human disease [249]. There are five human AAV serotypes. AAV-based vectors are usually based on serotype 1 or 2. They can transduce a wide variety of cells derived from different tissues [250], including nondividing cells [79, 85] and hematopoietic stem cells [251], although a substantial variability in transduction efficiency among different cell lines has been observed. In addition, AAV vectors may allow for stable transgene expression, following integration of the viral genome into the cellular chromosomal DNA [252]. Interestingly, wild-type AAV has the capability of integrating its genome specifically into the q arm of chromosome 19, between q13.3 and qter [80-84]. Unfortunately, this desirable property is not conserved in recombinant AAV vectors [5], raising the problem of possible insertional mutagenesis (Table 1). AAV has a small genome of 4.7 kb. The insertional capacity for foreign genes is limited to the range of about 4 kb. In some studies, transgenes had to be truncated to be adapted to the AAV vector system. The life cycle of AAV consists of two phases: latent infection and lytic phase. Entry into the lytic phase is caused by superinfection of latently infected cells, or by the coinfection of an adenovirus or a herpesvirus [253-255]. Recombinant AAV vector stocks are usually generated by infecting the AAV packaging cell line with an adenovirus. The lytic infection of AAV is triggered by the adenoviral early genes E1 and E4 [256]. Recombinant AAV vectors can be produced at high titers, such as 10^{10} cfu/ml, but it is rather difficult to obtain pure stocks that are helper virus-free. This poses a limitation to the application of AAV-mediated gene transfer in clinical trials. Recombinant AAV vector systems have been engineered following the same routes used for retroviral vectors. The AAV transfer vector is devoid of all the viral genes, except for two inverted terminal repeats (ITRs). The AAV transfer vector may contain either a marker gene or therapeutic factor. Another plasmid contains the AAV *rep* and *cap* genes, which encode for AAV packaging components and are under the control of a constitutive promoter. The ITRs have been removed from this plasmid. The highly transfectable 293 cell line is used as packaging cell line. The AAV transfer vector and the AAV packaging construct are simultaneously cotransfected in 293 cells, which are then infected with adenovirus in order to activate replication of the recombinant AAV vector. The mechanism of viral entry into the target cells has recently been elucidated for AAV type 2 [257, 258]. It was found that AAV type 2 first binds to heparan sulfate proteoglycan [259], and then binds to $\alpha V\beta 5$ integrin [257, 258]. This finding explains the broad cell tropism of AAV, and the variability observed in transduction efficiency among various cell lines, especially for primary human hematopoietic stem cells [260]. Neither low efficiency nor even the failure of transduction of certain target cells are originated by lack of AAV binding to the cell membrane [258]. For instance, the murine NIH3T3 cell line is refractory to AAV infection, despite efficient binding of the virus to the cell membrane via heparan sulfate proteoglycan receptor [258]. Therefore, the differential distribution of $\alpha V\beta 5$ AAV coreceptor among various cell lines accounts for the transduction efficiency variability observed for AAV-mediated gene transfer. The identification of the exact steps of viral entry may have important implications for optimizing gene transfer applications, apart from the prevention of viral infection. An example is the finding that expression of $\alpha V\beta 5$ integrin is an essential requirement for efficient adenoviral-mediated gene transfer to the human airway [236]. To this end, as already mentioned, adenoviral vectors based on serotype 5 have been genetically engineered to

express fibers of serotype 7, which bind to αVβ5 integrin with higher affinity than the fibers of serotype 5 [233, 234]. A study has demonstrated that the capsid of AAV type 2 can also be genetically modified by introduction of short foreign genes [261]. Specifically, a 14-amino-acid peptide containing a RGD motif was inserted into the AAV *cap* gene, in order to confer to the virion an altered cell tropism to infect cells refractory to AAV type 2 infection, such as mouse melanoma B16F10. The transduction efficiency of the chimeric AAV vector was estimated by *LacZ* reporter gene expression in B16F10 cells to be in the range of 10^4 cfu/ml [261]. Another report has generated a gross hybrid system by fusing in frame a single chain antibody with the AAV type 2 capsid to target the CD34 molecule [262]. The transduction efficiency of the chimeric AAV vector was about 10^2 cfu/ml in $CD34^+$ cells [262]. An aspect that is going to be investigated is the low efficiency of recombinant AAV vectors in transducing airway epithelial cells [263]. Epithelial cells express the αVβ5 integrin, which is the coreceptor for AAV type 2. Indeed, the levels of αVβ5 integrin expression allow for an efficient transduction of airway epithelial cells by adenoviral vectors carrying the fibers of serotype 7 [233, 234]. One hypothesis, which may account for the inefficient AAV-mediated gene transfer to airway epithelial cells, is that AAV vectors encounter a physical barrier of negatively charged molecules, such as mucins and glycosaminoglycans [263]. The improvement of AAV-mediated gene transfer to the airway epithelial cells has important implications for the development of gene therapy programs for the treatment of cystic fibrosis. Another study has tackled this problem by systematic administration of recombinant AAV vectors based either on type 2 or type 3 through bronchoscopic delivery in rabbits [264]. The results of this study have indicated that the procedure was safe, as no significant inflammatory responses were observed in rabbits. In addition, the transgene expression was efficient despite the level of neutralizing antibodies to AAV detected in the serum of rabbits [264].

The elucidation of the AAV type 2 entry mechanism into the target cells has been a breakthrough in the field of vector design. Besides providing useful information about how to improve the transduction efficiency for certain cell types, the identification of viral receptors may also be applied for the purification of recombinat AAV vectors, in order to obtain clinical-grade preparations [265].

Nonviral Vectors

Gene delivery systems based on nonviral vectors mainly comprise cationic liposomes [89-92, 272], DNA-protein complexes [93, 94] and mechanic administration of naked DNA [22, 26, 27, 30]. These systems are relatively easy to manipulate. Nonviral vectors are not infectious and are not very toxic. Furthermore, nonviral vectors allow for the delivery of large DNA fragments and are also particularly suitable to deliver oligonucleotides to mammalian cells, which is an excellent feature for the application of antisense strategies to downregulate the expression of certain genes (Table 1). Antisense strategies can be applied to a variety of pathologic conditions, including cancer, infectious diseases, and to prevent the rejection of allograft transplantation of organs [226]. In addition, as already mentioned, liposomes have been used to enhance the gene delivery efficiency of retroviral [147] and adenoviral vectors [244].

A number of obstacles have severely limited the application of nonviral-based vectors in therapy and preclinical studies [5]. The lack of specific targeting, the low transfection efficiency and the fact that transgene expression is only transient make difficult the in vivo applications of nonviral gene delivery systems. In addition, the unmethylated CpG islands of bacterial DNA elicit strong host immune responses [32, 266]. Furthermore, the liposome-mediated delivery of plasmid DNA enhances the immune responses to unmethylated CpG motifs more than the injection of plasmid DNA alone, probably because liposomes increase the cellular uptake of DNA. This is positive for genetic immunization purposes, but it is a considerable drawback to many other in vivo applications. The employment of cationic liposomes for the delivery of plasmid DNA was not succesful in the early gene therapy clinical trials for the treatment of cystic fibrosis, due to inflammatory reactions that were observed in the patients, which are also responsible for the inactivation of the transgene [267]. A recent study has circumvented this issue in an animal model by combining the administration of cationic liposomes with immunosuppressive drugs [268]. Other approaches may be based on the removal and/or methylation of CpG motifs in the plasmids, but this will cause the silencing of the promoter. The utilization of nonviral promoters has also been considered, but the alternative is mammalian housekeeping gene promoters, which are too weak.

Recent studies have reported significant success in improving the in vivo performance of nonviral gene delivery systems [18, 269-271]. These improvements have been achieved by developing new formulations of cationic liposomes, or of other composite nonviral vector systems. Interestingly, one study has generated an oral vaccine that was made of DNA nanoparticles complexed with chitosan, which is a natural biocompatible polysaccharide [18]. The aim of this study was to prevent peanut-induced anaphylaxis in a relevant murine model [18]. This goal was successfully achieved by oral administration of a DNA vaccine encoding for the *Arah2* gene, which is the main peanut allergen. The function of chitosan was to preserve the DNA until it reached the intestine [18]. A similar approach was adopted

in another study in which the plasmid DNA was complexed with atecollagen, a biocompatible polymer [269].

Conclusion

The improvement of vector design has allowed for a broader range of therapeutic applications for gene transfer technology. Gene therapy has a more active role in clinical trials, and there has been a dramatic increase in the number of preclinical studies for gene therapy and genetic immunization programs. However, the degree of vector development is still not sufficiently adequate to meet all the requirements for phase III clinical trials. The field of vector design has to address very difficult tasks from the standpoint of improvement of the transduction efficiency and safety precautions. The assessment of the risk/benefit ratio can be to some extent predicted only for limited cohorts of patients, who usually have poor clinical outcomes and short life expectancy at the time of the enrollment in gene therapy clinical trials. The application of gene-based interventions to other subjects is likely to be obstructed by an unfavorable risk/benefit ratio. In addition, the assessment of the risks associated with administration of viral-based vectors appears more complicated than previously thought. A number of complex issues must be addressed to evaluate the probability of having adverse effects in patients related to the treatment, and to establish the extent of the possible harm that patients may sustain. So far, the safety issues that have been considered are related to the immunogenicity of the vectors, the formation of replication-competent viruses in patients and the presence of contaminating agents in the vector preparations. Other pressing safety issues are related to insertional mutagenesis, possible recombinations between retroviral vectors and HERVs, and transmission of viral and other exogenous DNA to the germ line. To date, it is not easy to assess exactly all these risk factors. The nature of the risks associated with gene therapy treatments must be established and minimized as much as possible, in order to have a more positive risk/benefit ratio in favor of intervention. When all the requirements for more efficient gene delivery and safer therapeutic applications are met, gene transfer technology will become an accepted reality in the clinical setting.

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